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Priority application 17.05.1999



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Patentanmeldung Nr. Patent application No. Demande de brevet n°

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For the President of the European Patent Office

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**Blatt 2 der Bescheinigung  
Sheet 2 of the certificate  
Page 2 de l'attestation**

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Titre de l'invention:

**Adenovirus derived gene delivery vehicles comprising at least one element of adenovirus type 35**

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**SEE FOR TITLE PAGE 1 OF THE DESCRIPTION**

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Title: SEROTYPE OF ADENOVIRUS AND USES THEREOF

17. 05. 1999

The present invention relates to the field of gene therapy<sup>(58)</sup>, in particular gene therapy involving elements derived from viruses, more in particular elements of adenoviruses.

Adenoviruses have been proposed as suitable vehicles to deliver genes to the host.

There are a number of features of adenoviruses that make them particularly useful for the development of gene-transfer vectors for human gene therapy:

The adenovirus genome is well characterized. It consists of a linear double-stranded DNA molecule of approximately 36000 base pairs. The adenovirus DNA contains identical Inverted Terminal Repeats (ITR) of approximately 90-140 base pairs with the exact length depending on the serotype. The viral origins of replication are within the ITRs exactly at the genome ends;

The biology of the adenoviruses is characterized in detail; the adenovirus is not associated with severe human pathology in immuno-competent individuals

The virus is extremely efficient in introducing its DNA into the host cell; the virus can infect a wide variety of cells and has a broad host-range;

The virus can be produced at high virus titers in large quantities;

The virus can be rendered replication defective by deletion of the early-region 1 (E1) of the viral genome (Brody et al, 1994). Most adenoviral vectors currently used in gene therapy have a deletion in the E1 region, where desired genetic information can be introduced.

Based on these features, preferred methods for in vivo gene transfer into human target cells, make use of adenoviral vectors as gene delivery vehicles.

However, there are still drawbacks associated with the therapeutic use of adenoviral vectors in humans. A major

drawback is the existence of widespread pre-existing immunity among the population against adenoviruses. Exposure to wild-type adenoviruses is very common in humans, as has been documented extensively [reviewed in Wadell, 1984]. This exposure has resulted in immune responses against most types of adenoviruses, not alone against adenoviruses to which individuals have actually been exposed, but also against adenoviruses which have similar (neutralizing) epitopes. This phenomenon of pre-existing antibodies in humans, in combination with a strong secondary humoral and cellular immune response against the virus, can seriously affect gene transfer using recombinant adenoviral vectors.

To date, six different subgroups of human adenoviruses have been proposed which in total encompasses 51 distinct adenovirus serotypes (see table 1). A serotype is defined on the basis of its immunological distinctiveness as determined by quantitative neutralization with animal antisera (horse, rabbit). If neutralization shows a certain degree of cross-reaction between two viruses, distinctiveness of serotype is assumed if A) the hemagglutinins are unrelated, as shown by lack of cross-reaction on hemagglutination-inhibition, or B) substantial biophysical/ biochemical differences in DNA exist (Franki et al, 1991). The nine serotypes identified last (42-51) were isolated for the first time from HIV-infected patients (Hierholzer et al 1988; Schnurr et al 1993;). For reasons not well understood, most of such immune-compromised patients shed adenoviruses that were rarely or never isolated from immune-competent individuals (Hierholzer et al 1988, 1992; Khoo et al, 1995, De Jong et al, 1998).

The vast majority of individuals have had previous exposure to adenoviruses, especially the well investigated adenovirus serotypes 5 and type 2 (Ad5 and Ad2) or immunologically related serotypes. Importantly, these two serotypes are also the most extensively studied for use in human gene therapy.

As said before, the usefulness of these adenoviruses or cross-immunizing adenoviruses to prepare gene delivery vehicles may be seriously hampered, since the individual to which the gene delivery vehicle is provided, will raise a neutralising response to such a vehicle before long.

There is thus a need in the field of gene therapy to provide gene delivery vehicles, preferably based on adenoviruses, which do not encounter pre-existing immunity and/or which are capable of avoiding or diminishing neutralizing antibody responses.

Thus the invention provides a gene delivery vehicle comprising at least one of the adenovirus serotype 35 elements or a functional equivalent thereof, responsible for avoiding or diminishing neutralising activity against adenoviral elements by the host to which the gene is to be delivered and a gene of interest. A functional

equivalent/homologue of adenovirus 35 (element) for the purpose of the present invention is an adenovirus (element) which, like adenovirus 35, encounters pre-existing immunity in less than about 10% of the hosts to which it is administered for the first time, or which is capable in more than about 90% of the hosts to which it is administered to avoid or diminish the immune response. Typical examples of

~~such adenoviruses are adenovirus serotypes 34, 26 and 48. A~~

gene delivery vehicle may be based on adenovirus 35 or a functional homologue thereof, but it may also be based on another backbone, such as that of adenovirus 2 or 5, as long as it comprises at least one of the elements from adenovirus 35 or a functional equivalent thereof, which leads to the diminishing of the immune response against such an

adenovirus 2 or adenovirus 5 based gene delivery vehicle. Of course the gene delivery vehicle may also comprise elements from other (adeno) viruses, as long as one replaces an element which could lead to immunity against such a gene delivery vehicle by an element of adenovirus 35 or a

functional homologue thereof, which has less of such a drawback and which preferably avoids such a drawback. In the

present invention a gene delivery vehicle is any vehicle that is capable of delivering a nucleic acid of interest to a host cell. It must, according to the invention comprise an element of adenovirus 35 or a functional equivalent of such an element, which must have a beneficial effect regarding the immune response against such a vehicle. Basically all other elements making up the vehicle can be any elements known in the art or developed in the art, as long as together they are capable of delivering said nucleic acid of interest. In principle the person skilled in the art can use and/or produce any adenoviral products or production systems that can or have been applied in the adenoviral field. Typically the products of the invention can be made in the packaging cells useable for e.g. adenovirus 5, typically the vectors based on adenovirus 35 can be produced and/or used in the same manner as those of other adenoviruses e.g. adenovirus 2 and/or 5. A good overview of the possibilities of minimal vectors, packaging systems, intracellular amplification, vector and plasmid based systems can be found in applicant's copending applications (PCT/NL99/00235 and PCT/NL96/00244) incorporated herein by reference. Non-viral delivery systems can also be provided with elements according to the invention as can viral delivery systems. ....

Both kinds of systems are well known in the art in many different set-ups and do therefor not need any further elaboration here. A review on the many different systems and their properties can be found in Robbins and Ghivizzani (1998) and in Prince (1998) incorporated herein by reference. A nucleic acid of interest can be a gene or a functional part of a gene (wherein a gene is any nucleic acid which can be expressed) or a precursor of a gene or a transcribed gene on any nucleic acid level (DNA and/or RNA: double or single stranded). Genes of interest are well known in the art and typically include those encoding therapeutic proteins such as TPA, EPO, cytokines, antibodies or



derivatives thereof, etc. An overview of therapeutic proteins to be applied in gene therapy are listed below.

Immune-stimulatory factors like tumor-specific antigens, cytokines, etc.;

Anti-angiogenic factors non-limiting examples endostatin, angiostatin, ATF-BPTI CDT-6, dominant negative VEGF-mutants, etc.;

Angiogenic factors non-limiting example VEGF, Fibroblast growth factors, Nitric oxide synthases, C-type natriuretic peptide, etc.;

Inflammation inhibiting proteins like soluble CD40, FasL, IL-12, IL-10, IL-4, IL-13 and excreted single chain antibodies to CD4, CD5, CD7, CD52, IL-2, IL-1, IL-6, TNF, etc. or excreted single chain antibodies to the T-cell receptor on the auto-reactive T-cells. Also, dominant negative mutants of PML may be used to inhibit the immune response.

Furthermore, antagonists of inflammation promoting cytokines may be used, for example IL-1RA(receptor antagonist) and soluble receptors like sIL-1RI, sIL-1RII, sTNFR1 and sTNFR2. Growth and/or immune response inhibiting genes such as ~~ceNOS, Bcl3, cactus and I $\kappa$ B $\alpha$ ,  $\beta$ - or  $\gamma$  and apoptosis~~

inducing proteins like the VP3 protein of chicken anemia virus may also be used. Furthermore, suicide genes like HSV-TK, cytosine deaminase, nitroreductase and linamerase may be used.

A nucleic acid of interest may also be a nucleic acid which can hybridise with a nucleic acid sequence present in the host cell thereby inhibiting expression or transcription or translation of said nucleic acid. It may also block through cosuppression. In short a nucleic acid of interest is any nucleic acid that one may wish to provide a cell with in order to induce a response by that cell, which response may

be production of a protein, inhibition of such production, apoptosis, necrosis, proliferation, differentiation etc. The present invention is the first to disclose adenovirus 35 or a functional homologue thereof for therapeutical use, therefor the invention also provides an adenovirus serotype 35 or a functional homologue thereof or a chimaeric virus derived therefrom, or a gene delivery vehicle based on said virus its homologue or its chimaera for use as a pharmaceutical. The serotype of the present invention, adenovirus type 35, is in itself known in the art. It is an uncommon group B adenovirus that was isolated from patients with acquired immunodeficiency syndrome and other immunodeficiency disorders (Flomenberg et al., 1987; De Jong et al., 1983). Ad 35 has been shown to differ from the more fully characterized subgroup C (including Ad2 and Ad5) with respect to pathogenic properties (Basler et al., 1996). It has been suggested that this difference may be correlated with differences in the E3 region of the Ad35 genome (Basler et al., 1996). The DNA of Ad35 has been partially cloned and mapped (Kang et al., 1989a and b; Valderrama-Leon et al., 1985).

B type adenovirus serotypes such as 34 and 35 have a different E3 region than other serotypes. Typically this region is involved in suppressing immune response to adenoviral products. Thus the invention provides a gene delivery vehicle according to the invention whereby said elements involved in avoiding or diminishing immune response comprise adenovirus 35 E3 expression products or the genes encoding them or functional equivalents of either or both. Another part of adenoviruses involved in immune responses is the capsid, in particular the penton and/or the hexon proteins. Thus the invention also provides a gene delivery vehicle according to the invention whereby the elements comprise at least one adenovirus 35 capsid protein or functional part thereof, such as fiber, penton and/or hexon

proteins or a gene encoding at least one of them. It is not necessary that a whole protein relevant for immune response is of adenovirus 35 (or a functional homologue thereof) origin. It is very well possible to insert a part of an adenovirus fiber, penton or hexon protein into another fiber, penton or hexon. Thus chimaeric proteins are obtained.

It is also possible to have a penton of a certain adenovirus, a hexon from another and a fiber or an E3 region from yet another adenovirus. According to the invention at least one of the proteins or genes encoding them should comprise an element from adenovirus 35 or a functional homologue thereof, whereby said element has an effect on the immune response of the host. Thus the invention provides a gene delivery according to the invention, which is a chimaera of adenovirus 35 with at least one other adenovirus. In this way one can also modify the resulting virus in other aspects than the immune response alone. One can enhance its efficiency of infection with elements responsible therefor; one can enhance its replication on a packaging cell, or one can change its tropism.

Thus the invention e.g. provides a gene delivery vehicle according to the invention which has a different tropism

than adenovirus 35. Of course the tropism should be altered preferably such that the gene delivery vehicle is delivered preferentially to a subset of the host's cells, i.e. the target cells. Changes in tropism and other changes which can also be applied in the present invention of adenoviral or other gene delivery vehicles are disclosed in applicant's copending applications (nos. 98204482.8, 99200624.7 and 98202297.2) incorporated herein by reference. Of course the present application also provides any and all building blocks necessary and/or useful to get to the gene delivery vehicles and/or the chimaeras, etc. of the present invention. This includes packaging cells such as PER.C6 (ECACC deposit number 96022940) or cells based thereon, but

adapted for Ad35 or a functional homologue thereof; it also includes any nucleic acids encoding functional parts of adenovirus 35 or a functional homologue thereof, such as helper constructs and packaging constructs, as well as vectors comprising genes of interest and e.g. an ITR, etc. Typically applicant's application (PCT/NL96/00244) incorporated herein by reference, discloses elements necessary and useful for arriving at the invented gene delivery vehicles. Thus the invention also provides a nucleic acid encoding at least a functional part of a gene delivery vehicle according to the invention, or a virus, homologue or chimaera thereof according to the invention. According to the invention, such elements, which encode functions that will end up in the resulting gene delivery vehicle must comprise or be encoded by a nucleic acid encoding at least one of the adenovirus serotype 35 elements or a functional equivalent thereof, responsible for avoiding or diminishing neutralising activity against adenoviral elements by the host to which the gene is to be delivered. Typically the gene of interest would be present on the same nucleic acid which means that such a nucleic acid has such a gene or that it has a site for introducing a gene of interest therein.

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Typically such a nucleic acid also comprises at least one ITR and if it is a nucleic acid to be packaged also a packaging signal. However, as mentioned before all necessary and useful elements and/or building blocks for the present invention can be found in applicant's application (PCT/NL96/00244). A set of further improvements in the field of producing adenoviral gene delivery vehicles is applicant's plasmid system disclosed in PCT/NL99/00235 mentioned herein before. This system works in one embodiment as a homologous recombination of an adapter plasmid and a longer plasmid, together comprising all elements of the nucleic acid to be incorporated in the gene delivery vehicle. These methods can also be applied to the presently

invented gene delivery vehicles and their building elements. Thus the invention also provides a nucleic acid according to the invention further comprising a region of nucleotides designed or useable for homologous recombination, preferably as part of at least one set of two nucleic acids comprising a nucleic acid according to the invention, whereby said set of nucleic acids is capable of a single homologous recombination event with each other, which leads to a nucleic acid encoding a functional gene delivery vehicle.

Both empty packaging cells (in which the vector to be packaged to make a gene delivery vehicle according to the invention still has to be introduced or produced) as well as cells comprising a vector according to the invention to be packaged are provided. Thus the invention also encompasses a cell comprising a nucleic acid according to the invention or a set of nucleic acids according to the invention, preferably a cell which complements the necessary elements for adenoviral replication which are absent from the nucleic acid according to be packaged, or from a set of nucleic acids according to the invention.

Preferably such a cell originates from a PER.C6 cell (ECACC deposit number 96022940), or a similar packaging cell complementing with elements from Ad 35 or a functional

homologue thereof. As already alluded to the invention also encompasses a method for producing a gene delivery vehicle according to the invention, comprising expressing a nucleic acid according to the invention in a cell according to the invention and harvesting the resulting gene delivery vehicle. The above refers to the filling of the empty packaging cell with the relevant nucleic acids. The format of the filled cell is of course also part of the present invention, which provides a method for producing a gene delivery vehicle according to the invention, comprising culturing a filled packaging cell (producer cell) according to the invention in a suitable culture medium and harvesting the resulting gene delivery vehicle.

The resulting gene delivery vehicles obtainable by any method according to the invention are of course also part of the present invention, particularly also a gene delivery vehicle according to the invention, which is derived from a chimaera of an adenovirus and an integrating virus.

It is well known that adenoviral gene delivery vehicles do not integrate into the host genome normally. For long term expression of genes in a host cell it is therefor preferred to prepare chimaeras which do have that capability. Such

chimaeras have been disclosed in our copending application PCT/NL98/00731 incorporated herein by reference. A very good example of such a chimaera of an adenovirus and an

integrating virus wherein said integrating virus is an adeno associated virus. As discussed hereinbefore other useful

chimaeras, which can also be combined with the above are chimaeras (be it in swapping whole proteins or parts thereof or both) which have altered tropism. A very good example thereof is a chimaera of Ad 35 and Ad 16, possibly with

elements from for instance Ad 2 or Ad 5, wherein the tropism determining part of Ad 16 or a functional equivalent thereof is used to direct the gene delivery vehicle to synoviocytes and/or smooth muscle cells (see our copending applications

~~nos. 98204482.8 and 99200624.7) incorporated herein by reference).~~

The gene delivery vehicles according to the invention can be used to deliver genes or nucleic acids of interest to host cells. This will typically be a pharmaceutical use. Such a use is included in the present invention. Compositions suitable for such a use are also part of the present

invention. The amount of gene delivery vehicle that needs to be present per dose or per infection (m.o.i) will depend on the condition to be treated, the route of administration (typically parenteral) the subject and the efficiency of infection, etc. Dose finding studies are well known in the

art and those already performed with other (adenoviral) gene delivery vehicles can typically be used as guides to find

suitable doses of the gene delivery vehicles according to the invention. Typically this is also where one can find suitable excipients, suitable means of administration, suitable means of preventing infection with the vehicle where it is not desired, etc. Thus the invention also provides a pharmaceutical formulation comprising a gene delivery vehicle according to the invention and a suitable excipient, as well as a pharmaceutical formulation comprising an adenovirus, a chimaera thereof, or a functional homologue thereof according to the invention and a suitable excipient.

#### Detailed description

As described above, the most extensively studied serotypes of adenovirus are not ideally suitable for delivering additional genetic material to host cells. This is partly due to the pre-existing immunity among the population against these serotypes. This presence of pre-existing antibodies in humans, in combination with a strong secondary humoral and cellular immune response against the virus will affect adenoviral gene therapy.

The present invention provides the use of at least elements of a serotype and functional homologues thereof of adenovirus which are very suitable as gene therapy vectors. The present invention also discloses an automated high-throughput screening of all known adenovirus serotypes against sera from many individuals. Surprisingly, no neutralizing ability was found in any of the sera that were evaluated against one particular serotype, adenovirus 35 (Ad35). This makes the serotype of the present invention extremely useful as a vector system for gene therapy in man. Such vector system is capable of efficiently transferring genetic material to a human cell without the inherent problem of pre-existing immunity.

Typically, a virus is produced using an adenoviral vector (typically a plasmid, a cosmid or baculovirus vector). Such vectors are of course also part of the present invention. The invention also provides adenovirus derived vectors that  
5 have been rendered replication defective by deletion or inactivation of the E1 region. Of course, also a gene of interest can be inserted at for instance the site of E1 of the original adenovirus from which the vector is derived. In all aspects of the invention the adenoviruses may contain  
10 deletions in the E1 region and insertions of heterologous genes linked either or not to a promoter. Furthermore, the adenoviruses may contain deletions in the E2, E3 or E4 regions and insertions of heterologous genes linked to a promoter. In these cases, E2 and/or E4 complementing cell  
15 lines are required to generate recombinant adenoviruses.

One may choose to use the Ad35 serotype itself for the preparation of recombinant adenoviruses to be used in gene therapy. Alternatively, one may choose to use elements  
20 derived from the serotype of the present invention in such recombinant adenoviruses. One may for instance develop a chimaeric adenovirus that combines desirable properties from different serotypes. ~~Some serotypes have a somewhat limited~~ host range, but have the benefit of being less immunogenic,  
25 some are the other way round. Some have a problem of being of a limited virulence, but have a broad host range and/or a reduced immunogenicity. Such chimaeric adenoviruses are known in the art, and they are intended to be within the scope of the present invention. Thus in one embodiment the  
30 invention provides a chimaeric adenovirus comprising at least a part of the adenovirus genome of the present serotype, providing it with absence of pre-existing immunity, and at least a part of the adenovirus genome from another adenovirus serotype resulting in a chimaeric  
35 adenovirus. In this manner the chimaeric adenovirus produced is such that it combines the absence of pre-existing



immunity of the serotype of the present invention, to other characteristics of another serotype. Such characteristics may be temperature stability, assembly, anchoring, redirected infection, production yield, redirected or improved infection, stability of the DNA in the target cell, etc.

A packaging cell will generally be needed in order to produce sufficient amount of adenoviruses. For the production of recombinant adenoviruses for gene therapy purposes, several cell lines are available. These include but are not limited to the known cell lines PER.C6 (ECACC deposit number 96022940), 911, 293, and E1 A549.

An important feature of the present invention is the means to produce the adenovirus. Typically, one does not want an adenovirus batch for clinical applications to contain replication competent adenovirus. In general therefore, it is desired to omit a number of genes (but at least one) from the adenoviral genome on the adenoviral vector and to supply these genes in the genome of the cell in which the vector is brought to produce chimaeric adenovirus. Such a cell is usually called a packaging cell. The invention thus also provides a packaging cell for producing an adenovirus (a gene delivery vehicle) according to the invention,

comprising in trans all elements necessary for adenovirus production not present on the adenoviral vector according to the invention. Typically vector and packaging cell have to be adapted to one another in that they have all the necessary elements, but that they do not have overlapping elements which lead to replication competent virus by recombination.

Thus the invention also provides a kit of parts comprising a packaging cell according to the invention and a recombinant vector according the invention whereby there is essentially no sequence overlap leading to recombination resulting in

the production of replication competent adenovirus between said cell and said vector.

Thus the invention provides methods for producing  
5 adenovirus, which upon application will escape pre-existing humoral immunity, comprising providing a vector with elements derived from an adenovirus serotype against which virtually no natural immunity exists and transfecting said vector in a packaging cell according to the invention and  
10 allowing for production of viral particles.

In one aspect this invention describes the use of the adenovirus serotype of the present invention to overcome natural existing or induced, neutralising host activity  
15 towards adenoviruses administered in vivo for therapeutic applications. The need for a new serotype is stressed by observations that 1) repeated systemic delivery of recombinant adenovirus serotype 5 is unsuccessful due to formation of high titers of neutralising antibodies against  
20 the recombinant adenovirus serotype 5 (Schulick et al, 1997), and 2) pre-existing or humoral immunity is widespread in the population.

~~In another aspect this invention provides the use of gene~~  
delivery vehicles of the invention or the use of adenovirus  
25 serotype 35 for vaccination purposes. Such use prevents at least in part undesired immune responses of the host. Non-limiting examples of undesired immune responses are evoking an immune response against the gene delivery vehicle or adenovirus serotype 35 and/or boosting of an immune response  
30 against the gene delivery vehicle or adenovirus serotype 35. In another aspect of the invention, alternating use is made of Ad vectors belonging to different subgroups. This aspect of the invention therefore circumvents the inability to repeat the administration of an adenovirus for gene therapy  
35 purposes.

## Example 1

A high throughput assay for the detection of neutralising activity in human serum

5

To enable screening of a large amount of human sera for the presence of neutralising antibodies against all adenovirus serotypes, an automated 96-wells assay was developed.

*Human sera*

10

A panel of 100 individuals was selected. Volunteers (50% male, 50% female) were healthy individuals between 20 and 60 years old with no restriction for race. All volunteers signed an informed consent form. People professionally involved in adenovirus research were excluded.

15

Approximately 60 ml blood was drawn in dry tubes. Within two hours after sampling, the blood was centrifuged at 2500 rpm for 10 minutes. Approximately 30 ml serum was transferred to polypropylene tubes and stored frozen at -20°C until further use.

20

Serum was thawed and heat-inactivated at 56°C for 10 minutes and then aliquotted to prevent repeated cycles of freeze/thawing. Part was used to make five steps of twofold dilutions in medium (DMEM, Gibco BRL) in a quantity enough

25

to fill out approximately 70 96-well plates. Aliquots of undiluted and diluted sera were pipetted in deep well plates (96-well format) and using a programmed platemate dispensed in 100 µl aliquots into 96-well plates. This way the plates were loaded with eight different sera in duplo (100 µl/well)

30

according to the scheme below:

S1/ 2	S1/ 4	S1/ 8	S1/ 16	S1/ 32	S5/ 2	S5/ 4	S5/ 8	S5/ 16	S5/ 32	-	-
S1/ 2	S1/ 4	S1/ 8	S1/ 16	S1/ 32	S5/ 2	S5/ 4	S5/ 8	S5/ 16	S5/ 32	-	-
S2/ 2	S2/ 4	S2/ 8	S2/ 16	S2/ 32	S6/ 2	S6/ 4	S6/ 8	S6/ 16	S6/ 32	-	-
S2/ 2	S2/ 4	S2/ 8	S2/ 16	S2/ 32	S6/ 2	S6/ 4	S6/ 8	S6/ 16	S6/ 32	-	-
S3/ 2	S3/ 4	S3/ 8	S3/ 16	S3/ 32	S7/ 2	S7/ 4	S7/ 8	S7/ 16	S7/ 32	-	-
S3/ 2	S3/ 4	S3/ 8	S3/ 16	S3/ 32	S7/ 2	S7/ 4	S7/ 8	S7/ 16	S7/ 32	-	-
S4/ 2	S4/ 4	S4/ 8	S4/ 16	S4/ 32	S8/ 2	S8/ 4	S8/ 8	S8/ 16	S8/ 32	-	-
S4/ 2	S4/ 4	S4/ 8	S4/ 16	S4/ 32	S8/ 2	S8/ 4	S8/ 8	S8/ 16	S8/ 32	-	-

- Where S1/2 to S8/2 in columns 1 and 6 represent 1x diluted sera and Sx/4, Sx/8, Sx/16 and Sx/32 the twofold serial dilutions. The last plates also contained four wells filled with 100  $\mu$ l foetal calf serum as a negative control. Plates were kept at -20°C until further use.

#### *Preparation of human adenovirus stocks*

- 10 Prototypes of all known human adenoviruses were inoculated on T25 flasks seeded with PER.C6 cells (ECACC deposit number 96022940) (Fallaux et al., 1998) and harvested upon full CPE. After freeze/thawing 1-2 ml of the crude lysates was used to inoculate a T80 flask with PER.C6 cells (ECACC deposit number 96022940) and virus was harvested at full CPE. The timeframe between inoculation and occurrence of CPE as well as the amount of virus needed to re-infect a new culture, differed between serotypes. Adenovirus stocks were prepared by freeze/thawing and used to inoculate 3-4 T175 cm<sup>3</sup> three-layer flasks with PER.C6 cells (ECACC deposit number 96022940). Upon occurrence of CPE, cells were harvested by tapping the flask, pelleted and virus was isolated and purified by a two step CsCl gradient as follows. Cell pellets were dissolved in 50 ml 10 mM NaPO<sub>4</sub> buffer (pH 7.2) and frozen at -20°C. After thawing at 37°C,

- 5.6 ml sodium deoxycholate (5% w/v) was added. The solution was mixed gently and incubated for 5-15 minutes at 37°C to completely lyse the cells. After homogenizing the solution, 1875  $\mu$ l 1M MgCl<sub>2</sub> was added. After the addition of 375  $\mu$ l
- 5 DNase (10 mg/ml) the solution was incubated for 30 minutes at 37°C. Cell debris was removed by centrifugation at 1880xg for 30 minutes at RT without brake. The supernatant was subsequently purified from proteins by extraction with freon (3x). The cleared supernatant was loaded on a 1M Tris/HCl
- 10 buffered cesiumchloride blockgradient (range: 1.2/1.4 gr/ml) and centrifugated at 21000 rpm for 2.5 hours at 10°C. The virus band is isolated after which a second purification using a 1M Tris/HCl buffered continuous gradient of 1.33 gr/ml of cesiumchloride was performed. The virus was then
- 15 centrifuged for 17 hours at 55000 rpm at 10°C. The virus band is isolated and sucrose (50 % w/v) is added to a final concentration of 1%. Excess cesiumchloride is removed by dialysis (three times 1 hr at RT) in dialysis slides (Slide-a-lizer, cut off 10000 kDa, Pierce, USA) against 1.5 ltr PBS
- 20 supplemented with CaCl<sub>2</sub> (0.9 mM), MgCl<sub>2</sub> (0.5mM) and an increasing concentration of sucrose (1, 2, 5%). After dialysis, the virus is removed from the slide-a-lizer after
- which it is aliquoted in portions of 25 and 100  $\mu$ l upon which the virus is stored at -85°C.
- 25 To determine the number of virus particles per milliliter, 50  $\mu$ l of the virus batch is run on a high-pressure liquid chromatograph (HPLC) as described by Shabram et al (1997). Viruses were eluted using an NaCl gradient ranging from 0 to 600 mM. As depicted in table I, the NaCl concentration by
- 30 which the viruses were eluted differed significantly among serotypes.

Most human adenoviruses replicated well on PER.C6 cells ((ECACC deposit number 96022940) with a few exceptions. Adenovirus type 8 and 40 were grown on 911-E4

35 cells (He et al., 1998). Purified stocks contained between  $5 \times 10^{10}$  and  $5 \times 10^{12}$  virus particles/ml (VP/ml; see table I).

### *Titration of purified human adenovirus stocks*

Adenoviruses were titrated on PER.C6 cells (ECACC deposit number 96022940) to determine the amount of virus necessary to obtain full CPE in five days, the length of the neutralisation assay. Hereto, 100  $\mu$ l medium was dispensed into each well of 96-well plates. 25  $\mu$ l of adenovirus stocks prediluted  $10^4$ ,  $10^5$ ,  $10^6$  or  $10^7$  times were added to column 2 of a 96-well plate and mixed by pipetting up and down 10 times. Then 25  $\mu$ l was brought from column 2 to column 3 and again mixed. This was repeated until column 11 after which 25  $\mu$ l from column 11 was discarded. This way serial dilutions in steps of 5 were obtained starting off from a prediluted stock. Then  $3 \times 10^4$  PER.C6 cells (ECACC deposit number 96022940) were added in a 100  $\mu$ l volume and the plates were incubated at 37 °C, 5% CO<sub>2</sub> for five or six days. CPE was monitored microscopically. The method of Reed and Muensch was used to calculate the cell culture inhibiting dose 50% (CCID50).

In parallel identical plates were set up that were analysed using the MTT assay (Promega). In this assay living cells are quantified by colorimetric staining. Hereto, 20  $\mu$ l MTT (7.5 mgr/ml in PBS) was added to the wells and incubated at 37 °C, 5% CO<sub>2</sub> for two hours. The supernatant was removed and 100  $\mu$ l of a 20:1 isopropanol/triton-X100 solution was added to the wells. The plates were put on a 96-wells shaker for 3-5 minutes to solubilise precipitated staining. Absorbance was measured at 540 nm and at 690 nm (background). By this assay wells with proceeding CPE or full CPE can be distinguished.

### *Neutralisation assay*

96-well plates with diluted human serum samples were thawed at 37 °C, 5% CO<sub>2</sub>. Adenovirus stocks diluted to 200 CCID50 per 50  $\mu$ l were prepared and 50  $\mu$ l aliquots were added to columns 1-11 of the plates with serum. Plates were incubated

for 1 hour at 37°C, 5% CO<sub>2</sub>. Then 50 µl PER.C6 cells (ECACC deposit number 96022940) at 6x10<sup>5</sup>/ml were dispensed in all wells and incubated for 1 day at 37 °C, 5% CO<sub>2</sub>. Supernatant was removed using fresh pipet tips for each row and 200 µl fresh medium was added to all wells to avoid toxic effects of the serum. Plates were incubated for another 4 days at 37 °C, 5% CO<sub>2</sub>. In addition, parallel control plates were set up in duplo with diluted positive control sera generated in rabbits and specific for each serotype to be tested in rows A and B and with negative control serum (FCS) in rows C and D. Also, in each of the rows E-H a titration was performed as described above with steps of five times dilutions starting with 200 CCID<sub>50</sub> of each virus to be tested. On day 5 one of the control plates was analysed microscopically and with the MTT assay. The experimental titer was calculated from the control titration plate observed microscopically. If CPE was found to be complete, i.e. the first dilution in the control titration experiment analysed by MTT shows clear cell death, all assay plates were processed. If not, the assay was allowed to proceed for one or more days until full CPE was apparent after which all plates were processed. In most cases the assay was terminated at day 5. For Ad1, 5, 33, 39, 42 and 43 the assay was left for six days and for Ad2 for eight days.

A serum sample is regarded to be non-neutralising when at the highest serum concentration a maximum protection is seen of 40% compared to the controls without serum. The results of the analysis of 44 prototype adenoviruses against serum from 100 healthy volunteers is shown in figure 1. As expected the percentage of serum samples that contained neutralising antibodies to Ad2 and Ad5 was very high. This was also true for most of the lower numbered adenoviruses. Surprisingly, none of the serum samples contained neutralising antibodies to adenovirus serotype 35. Also, the number of individuals with neutralising antibody titers to the serotypes 26, 34 and 48 was very low.

Therefore, recombinant E1-deleted adenoviruses based on Ad35 or one of the other above mentioned serotypes have an important advantage compared to recombinant vectors based on Ad5 with respect to clearance of the viruses by neutralising antibodies.

Also, Ad5-based vectors that have (parts of) the capsid proteins involved in immunogenic response of the host replaced by the corresponding (parts of) the capsid proteins of Ad35 or one of the other serotypes will be less, or even not, neutralised by the vast majority of human sera. As can be seen in table I the VP/CCID50 ratio calculated from the virus particles per ml and the CCID50 obtained for each virus in the experiments was highly variable and ranged from 0.4 to 5 log. This is probably caused by different infection efficiencies of PER.C6 cells (ECACC deposit number 96022940) and by differences in replication efficiency of the viruses. Furthermore, differences in batch qualities may play a role. A high VP/CCID50 ratio means that more virus was put in the wells to obtain CPE in 5 days. As a consequence the outcome of the neutralisation study might be biased since more (inactive) virus particles could shield the antibodies. To check whether this phenomenon had taken place, the VP/CCID50 ratio was plotted against the percentage of serum samples found positive in the assay (Figure 2). The graph clearly shows that there is no negative correlation between the amount of viruses in the assay and neutralisation in serum.



**Example 2**

5 Generation of Ad5 plasmid vectors for the production of  
recombinant viruses and easy manipulation of adenoviral  
genes

*pBr/Ad.Bam-rITR (ECACC deposit P97082122)*

In order to facilitate blunt end cloning of the ITR  
sequences, wild-type human adenovirus type 5 (Ad5) DNA was  
10 treated with Klenow enzyme in the presence of excess dNTPs.  
After inactivation of the Klenow enzyme and purification by  
phenol/chloroform extraction followed by ethanol  
precipitation, the DNA was digested with BamHI. This DNA  
preparation was used without further purification in a  
15 ligation reaction with pBr322 derived vector DNA prepared as  
follows: pBr322 DNA was digested with EcoRV and BamHI,  
dephosphorylated by treatment with TSAP enzyme (Life  
Technologies) and purified on LMP agarose gel (SeaPlaque  
GTG). After transformation into competent *E.coli* DH5 $\alpha$  (Life  
20 Techn.) and analysis of ampiciline resistant colonies, one  
clone was selected that showed a digestion pattern as  
expected for an insert extending from the BamHI site in Ad5  
to the right ITR.

Sequence analysis of the cloning border at the right ITR  
25 revealed that the most 3' G residue of the ITR was missing,  
the remainder of the ITR was found to be correct. Said  
missing G residue is complemented by the other ITR during  
replication.

30 *pBr/Ad.Sal-rITR (ECACC deposit P97082119)*

*pBr/Ad.Bam-rITR* was digested with BamHI and Sali. The vector  
fragment including the adenovirus insert was isolated in LMP  
agarose (SeaPlaque GTG) and ligated to a 4.8 kb Sali-BamHI  
fragment obtained from wt Ad5 DNA and purified with the  
35 GeneClean II kit (Bio 101, Inc.). One clone was chosen and  
the integrity of the Ad5 sequences was determined by

restriction enzyme analysis. Clone pBr/Ad.Sal-rITR contains adeno type 5 sequences from the SalI site at bp 16746 up to and including the rITR (missing the most 3' G residue).

5 pBr/Ad.Cla-Bam (ECACC deposit P97082117)

wt Adeno type 5 DNA was digested with ClaI and BamHI, and the 20.6 kb fragment was isolated from gel by electro-elution. pBr322 was digested with the same enzymes and purified from agarose gel by GeneClean. Both fragments were  
10 ligated and transformed into competent DH5α. The resulting clone pBr/Ad.Cla-Bam was analysed by restriction enzyme digestion and shown to contain an insert with adenovirus sequences from bp 919 to 21566.

15 pBr/Ad.AflIII-Bam (ECACC deposit P97082114)

Clone pBr/Ad.Cla-Bam was linearized with EcoRI (in pBr322) and partially digested with AflIII. After heat inactivation of AflIII for 20' at 65°C the fragment ends were filled in with Klenow enzyme. The DNA was then ligated to a blunt  
20 double stranded oligo linker containing a PacI site (5'-AATTGTC~~CTTAATTA~~ACCGCTTAA-3'). This linker was made by annealing the following two oligonucleotides: 5'-AATTGTCTTAATTAACCGC-3' and 5'-AATTGCGGTTAATTAAGAC-3',  
followed by blunting with Klenow enzyme. After precipitation  
25 of the ligated DNA to change buffer, the ligations were digested with an excess PacI enzyme to remove concatameres of the oligo. The 22016 bp partial fragment containing Ad5 sequences from bp 3534 up to 21566 and the vector sequences, was isolated in LMP agarose (SeaPlaque GTG), religated and  
30 transformed into competent DH5α. One clone that was found to contain the PacI site and that had retained the large adeno fragment was selected and sequenced at the 5' end to verify correct insertion of the PacI linker in the (lost) AflIII site.

pBr/Ad.Bam-rITRpac#2 (ECACC deposit P97082120) and  
pBr/Ad.Bam-rITRpac#8 (ECACC deposit P97082121)

To allow insertion of a PacI site near the ITR of Ad5 in  
clone pBr/Ad.Bam-rITR about 190 nucleotides were removed  
5 between the ClaI site in the pBr322 backbone and the start  
of the ITR sequences. This was done as follows: pBr/Ad.Bam-  
rITR was digested with ClaI and treated with nuclease Bal31  
for varying lengths of time (2', 5', 10' and 15'). The  
extend of nucleotide removal was followed by separate  
10 reactions on pBr322 DNA (also digested at the ClaI site),  
using identical buffers and conditions. Bal31 enzyme was  
inactivated by incubation at 75 °C for 10', the DNA was  
precipitated and resuspended in a smaller volume TE buffer.  
To ensure blunt ends, DNAs were further treated with T4 DNA  
15 polymerase in the presence of excess dNTPs. After digestion  
of the (control) pBr322 DNA with SalI, satisfactory  
degradation (~150 bp) was observed in the samples treated  
for 10' or 15'. The 10' or 15' treated pBr/Ad.Bam-rITR  
samples were then ligated to the above described blunted  
20 PacI linkers (See pBr/Ad.AflIII-Bam). Ligations were purified  
by precipitation, digested with excess PacI and separated  
from the linkers on an LMP agarose gel. After religation,  
~~DNAs were transformed into competent DH5 $\alpha$  and colonies~~  
analyzed. Ten clones were selected that showed a deletion of  
25 approximately the desired length and these were further  
analyzed by T-track sequencing (T7 sequencing kit, Pharmacia  
Biotech). Two clones were found with the PacI linker  
inserted just downstream of the rITR. After digestion with  
PacI, clone #2 has 28 bp and clone #8 has 27 bp attached to  
30 the ITR.

pWE/Ad.AflIII-rITR (ECACC deposit P97082116)

Cosmid vector pWE15 (Clontech) was used to clone larger Ad5  
inserts. First, a linker containing a unique PacI site was  
35 inserted in the EcoRI sites of pWE15 creating pWE.pac. To  
this end, the double stranded PacI oligo as described for

pBr/Ad.AflIII-BamHI was used but now with its EcoRI protruding ends. The following fragments were then isolated by electro-elution from agarose gel: pWE.pac digested with PacI, pBr/AflIII-Bam digested with PacI and BamHI and

5 pBr/Ad.Bam-rITR#2 digested with BamHI and PacI. These fragments were ligated together and packaged using  $\lambda$  phage packaging extracts (Stratagene) according to the manufacturer's protocol. After infection into host bacteria, colonies were grown on plates and analyzed for presence of

10 the complete insert. pWE/Ad.AflIII-rITR contains all adenovirus type 5 sequences from bp 3534 (AflIII site) up to and including the right ITR (missing the most 3' G residue).

*pBr/Ad.lITR-Sal(9.4) (ECACC deposit P97082115)*

15 Adeno 5 wt DNA was treated with Klenow enzyme in the presence of excess dNTPs and subsequently digested with SalI. Two of the resulting fragments, designated left ITR-Sal(9.4) and Sal(16.7)-right ITR, respectively, were isolated in LMP agarose (Seaplaque GTG). pBr322 DNA was

20 digested with EcoRV and SalI and treated with phosphatase (Life Technologies). The vector fragment was isolated using the GeneClean method (BIO 101, Inc.) and ligated to the Ad5 SalI fragments. Only the ligation with the 9.4 kb fragment

gave colonies with an insert. After analysis and sequencing

25 of the cloning border a clone was chosen that contained the full ITR sequence and extended to the SalI site at bp 9462.

*pBr/Ad.lITR-Sal(16.7) (ECACC deposit P97082118)*

pBr/Ad.lITR-Sal(9.4) is digested with SalI and

30 dephosphorylated (TSAP, Life Technologies). To extend this clone upto the third SalI site in Ad5, pBr/Ad.Cla-Bam was linearized with BamHI and partially digested with SalI. A 7.3 kb SalI fragment containing adenovirus sequences from 9462-16746 was isolated in LMP agarose gel and ligated to

35 the SalI-digested pBr/Ad.lITR-Sal(9.4) vector fragment.

*pWE/Ad.AflIII-EcoRI*

pWE.pac was digested with ClaI and 5' protruding ends were filled using Klenow enzyme. The DNA was then digested with PacI and isolated from agarose gel. pWE/AflIII-rITR was digested with EcoRI and after treatment with Klenow enzyme digested with PacI. The large 24 kb fragment containing the adenoviral sequences was isolated from agarose gel and ligated to the ClaI-digested and blunted pWE.pac vector using the Ligation Express<sup>™</sup> kit from Clontech. After transformation of Ultracompetent XL10-Gold cells from Stratagene, clones were identified that contained the expected insert. pWE/AflIII-EcoRI contains Ad5 sequences from bp 3534-27336.

*Generation of pWE/Ad.AflIII-rITRsp*

The 3' ITR in the vector pWE/Ad.AflIII-rITR does not include the terminal G-nucleotide. Furthermore, the PacI site is located almost 30 bp from the right ITR. Both these characteristics may decrease the efficiency of virus generation due to inefficient initiation of replication at the 3' ITR. Note that during virus generation the left ITR in the adapter plasmid is intact and enables replication of the virus DNA after homologous recombination.

To improve the efficiency of initiation of replication at the 3' ITR, the pWE/Ad.AflIII-rITR was modified as follows: construct pBr/Ad.Bam-rITRpac#2 was first digested with PacI and then partially digested with AvrII and the 17.8 kb vector containing fragment was isolated and dephosphorylated using SAP enzyme (Boehringer Mannheim). This fragment lacks the adenosequences from nucleotide 35464 to the 3' ITR. Using DNA from pWE/Ad.AflIII-rITR as template and the primers ITR-EPH:

5'-CGG AAT TCT TAA TTA AGT TAA CAT CAT CAA TAA TAT ACC-3'

and

Ad101: 5'-TGA TTC ACA TCG GTC AGT GC-3'

a 630 bp PCR fragment was generated corresponding to the 3' Ad5 sequences. This PCR fragment was subsequently cloned in the vector pCR2.1 (Invitrogen) and clones containing the PCR fragment were isolated and sequenced to check correct amplification of the DNA. The PCR clone was then digested with PacI and AvrII and the 0.5 kb adeno insert was ligated to the PacI/ partial AvrII digested pBr/Ad.Bam-rITRpac#2 fragment generating pBr/Ad.Bam-rITRsp. Next this construct was used to generate a cosmid clone (as described above) that has an insert corresponding to the adenosequences 3534 to 35938. This clone was named pWE/AflIII-rITRsp.

*Generation of pWE/Ad.AflIII-rITRAE2A:*

Deletion of the E2A coding sequences from pWE/Ad.AflIII-rITR (ECACC deposit P97082116) has been accomplished as follows. The adenoviral sequences flanking the E2A coding region at the left and the right site were amplified from the plasmid pBr/Ad.Sal.rITR (ECACC deposit P97082119) in a PCR reaction with the Expand PCR system (Boehringer) according to the manufacturers protocol. The following primers were used: Right flanking sequences (corresponding Ad5 nucleotides 24033 to 25180):

ΔE2A.SnaBI: 5'-GGC GTA CGT AGC CCT GTC GAA AG-3'

ΔE2A.DBP-start: 5'-CCA ATG CAT TCG AAG TAC TTC CTT  
CTC CTA TAG GC-3'

The amplified DNA fragment was digested with SnaBI and NsiI (NsiI site is generated in the primer ΔE2A.DBP-start, underlined).

Left flanking sequences (corresponding Ad5 nucleotides 21557 to 22442):

ΔE2A.DBP-stop: 5'-CCA ATG CAT ACG GCG CAG ACG G-3'

ΔE2A.BamHI: 5'-GAG GTG GAT CCC ATG GAC GAG-3'

The amplified DNA was digested with BamHI and NsiI (NsiI site is generated in the primer ΔE2A.DBP-stop, underlined).

Subsequently, the digested DNA fragments were ligated into

SnaBI/BamHI digested pBr/Ad.Sal-rITR. Sequencing confirmed the exact replacement of the DBP coding region with a unique NsiI site in plasmid pBr/Ad.Sal-rITRAE2A. The unique NsiI site can be used to introduce an expression cassette for a gene to be transduced by the recombinant vector.

The deletion of the E2A coding sequences was performed such that the splice acceptor sites of the 100K encoding L4-gene at position 24048 in the top strand was left intact. In addition, the poly adenylation signals of the original E2A-RNA and L3-RNAs at the left hand site of the E2A coding sequences were left intact. This ensures proper expression of the L3-genes and the gene encoding the 100K L4-protein during the adenovirus life cycle.

Next, the plasmid pWE/Ad.AflIII-rITRAE2A was generated. The plasmid pBr/Ad.Sal-rITRAE2A was digested with BamHI and SpeI. The 3.9-Kb fragment in which the E2A coding region was replaced by the unique NsiI site was isolated. The pWE/Ad.AflIII-rITR was digested with BamHI and SpeI. The 35 Kb DNA fragment, from which the BamHI/SpeI fragment containing the E2A coding sequence was removed, was isolated. The fragments were ligated and packaged using  $\lambda$  phage-packaging extracts according to the manufacturer protocol (Stratagene), yielding the plasmid-pWE/Ad.AflIII-rITRAE2A.

This cosmid clone can be used to generate adenoviral vectors that are deleted for E2A by cotransfection of PacI digested DNA together with digested adapter plasmids onto packaging cells that express functional E2A gene product.

### 30 Construction of adapter plasmids

The absence of sequence overlap between the recombinant adenovirus and E1 sequences in the packaging cell line is essential for safe, RCA-free generation and propagation of new recombinant viruses. The adapter plasmid pMLPI.TK (described in PCT/NL96/00244) is an example of an adapter plasmid designed for use according to the invention in

combination with the improved packaging cell lines of the invention. This plasmid was used as the starting material to make a new vector in which nucleic acid molecules comprising specific promoter and gene sequences can be easily  
 5 exchanged.

First, a PCR fragment was generated from pZipΔMo+PyF101(N<sup>-</sup>) template DNA (described in PCT/NL96/00195) with the following primers: LTR-1: 5'-CTG TAC GTA CCA GTG CAC TGG CCT AGG CAT GGA AAA ATA CAT AAC TG-3' and LTR-2: 5'-GCG GAT CCT  
 10 TCG AAC CAT GGT AAG CTT GGT ACC GCT AGC GTT AAC CGG GCG ACT CAG TCA ATC G-3'. Pwo DNA polymerase (Boehringer Mannheim) was used according to manufacturers protocol with the following temperature cycles: once 5' at 95°C; 3' at 55°C; and 1' at 72°C, and 30 cycles of 1' at 95°C, 1' at 60°C, 1'  
 15 at 72°C, followed by once 10' at 72°C. The PCR product was then digested with BamHI and ligated into pMLP10 (Levrero et al., 1991) vector digested with PvuII and BamHI, thereby generating vector pLTR10. This vector contains adenoviral sequences from bp 1 up to bp 454 followed by a promoter  
 20 consisting of a part of the Mo-MuLV LTR having its wild-type enhancer sequences replaced by the enhancer from a mutant polyoma virus (PyF101). The promoter fragment was designated L420. Next, the coding region of the murine HSA gene was

inserted. pLTR10 was digested with BstBI followed by Klenow  
 25 treatment and digestion with NcoI. The HSA gene was obtained by PCR amplification on pUC18-HSA (Kay et al., 1990) using the following primers: HSA1, 5'-GCG CCA CCA TGG GCA GAG CGA TGG TGG C-3' and HSA2, 5'-GTT AGA TCT AAG CTT GTC GAC ATC GAT CTA CTA ACA GTA GAG ATG TAG AA-3'. The 269 bp amplified  
 30 fragment was subcloned in a shuttle vector using the NcoI and BglII sites. Sequencing confirmed incorporation of the correct coding sequence of the HSA gene, but with an extra TAG insertion directly following the TAG stop codon. The coding region of the HSA gene, including the TAG duplication  
 35 was then excised as a NcoI(sticky)-SalI(blunt) fragment and



cloned into the 3.5 kb NcoI(sticky)/BstBI(blunt) fragment from pLTR10, resulting in pLTR-HSA10.

Finally, pLTR-HSA10 was digested with EcoRI and BamHI after which the fragment containing the left ITR, packaging  
 5 signal, L420 promoter and HSA gene was inserted into vector pMLPI.TK digested with the same enzymes and thereby replacing the promoter and gene sequences. This resulted in the new adapter plasmid pAd/L420-HSA that contains  
 10 convenient recognition sites for various restriction enzymes around the promoter and gene sequences. SnaBI and AvrII can be combined with HpaI, NheI, KpnI, HindIII to exchange promoter sequences, while the latter sites can be combined with the ClaI or BamHI sites 3' from HSA coding region to replace genes in this construct.

15 Another adapter plasmid that was designed to allow easy exchange of nucleic acid molecules was made by replacing the promoter, gene and poly A sequences in pAd/L420-HSA with the CMV promoter, a multiple cloning site, an intron and a poly-A signal. For this purpose, pAd/L420-HSA was digested with  
 20 AvrII and BglII followed by treatment with Klenow to obtain blunt ends. The 5.1 kb fragment with pBr322 vector and adenoviral sequences was isolated and ligated to a blunt 1570 bp fragment from pCDNA1/amp (Invitrogen) obtained by

digestion with HhaI and AvrII followed by treatment with T4  
 25 DNA polymerase. This adapter plasmid was named pAd5/CLIP. To enable removal of vector sequences from the left ITR in pAd5/Clip, this plasmid was partially digested with EcoRI and the linear fragment was isolated. An oligo of the sequence 5' TTAAGTCGAC-3' was annealed to itself resulting  
 30 in a linker with a SalI site and EcoRI overhang. The linker was ligated to the partially digested pAd5/Clip vector and clones were selected that had the linker inserted in the EcoRI site 23 bp upstream of the left adenovirus ITR in pAd5/Clip resulting in pAd5/Clipsal. Likewise, the EcoRI  
 35 site in pAd5/Clip has been changed to a PacI site by insertion of a linker of the sequence 5'-

AATTGTCTTAATTAACCGCAATT-3'. The pAd5/Clip vector was partially digested with EcoRI, dephosphorylated and ligated to the PacI linker with EcoRI overhang. The ligation mixture was digested with PacI to remove concatamers, isolated from agarose gel and religated. The resulting vector was named pAd5/Clippac. These changes enable more flexibility to liberate the left ITR from the plasmid vector sequences. The vector pAd5/L420-HSA was also modified to create a Sall or PacI site upstream of the left ITR. Hereto pAd5/L420-HSA was digested with EcoRI and ligated to the above described PacI linker. The ligation mixture was digested with PacI and religated after isolation of the linear DNA from agarose gel to remove concatamerised linkers. This resulted in adapter plasmid pAd5/L420-HSApac. This construct was used to generate pAd5/L420-HSAsal as follows: pAd5/L420-HSApac was digested with ScaI and BsrGI and the vector fragment was ligated to the 0.3 kb fragment isolated after digestion of pAd5/Clipsal with the same enzymes.

#### 20 *Generation of adapter plasmids pAdMire and pAdApt*

To create an adapter plasmid that only contains a polylinker sequence and no promoter or polyA sequences, pAd5/L420-HSApac was digested with AvrII and BglII. The vector fragment was ligated to a linker oligonucleotide digested with the same restriction enzymes. The linker was made by annealing oligos of the following sequence:

PLL-1: 5'- GCC ATC CCT AGG AAG CTT GGT ACC GGT GAA TTC GCT AGC GTT AAC GGA TCC TCT AGA CGA GAT CTG G-3' and

PLL-2: 5'- CCA GAT CTC GTC TAG AGG ATC CGT TAA CGC TAG CGA ATT CAC CGG TAC CAA GCT TCC TAG GGA TGG C-3'.

The annealed linkers were digested with AvrII and BglII and separated from small ends by column purification (Qiaquick nucleotide removal kit) according to manufacturer's recommendations. The linker was then ligated to the AvrII/BglII digested pAd5/L420-HSApac fragment. A clone,

named AdMire, was selected that had the linker incorporated and was sequenced to check the integrity of the insert. Adapter plasmid AdMire enables easy insertion of complete expression cassettes.

- 5 An adapter plasmid containing the human CMV promoter that mediates high expression levels in human cells was constructed as follows: pAd5/L420-HSApac was digested with AvrII and 5' protruding ends were filled in using Klenow enzyme. A second digestion with HindIII resulted in removal
- 10 of the L420 promoter sequences. The vector fragment was isolated and ligated to a PCR fragment containing the CMV promoter sequence. This PCR fragment was obtained after amplification of CMV sequences from pCMVLacI (Stratagene) with the following primers:
- 15 CMVplus: 5'-GATCGGTACCACTGCAGTGGTCAATATTGGCCATTAGCC-3' and CMVminA: 5'-GATCAAGCTTCCAATGCACCGTTCCTGGC-3'.
- The PCR fragment was first digested with PstI (underlined in CMVplus) after which the 3'-protruding ends were removed by treatment with T4 DNA polymerase. Then the DNA was digested
- 20 with HindIII (underlined in CMVminA) and ligated into the above described pAd5/L420-HSApac vector fragment digested with AvrII and HindIII. The resulting plasmid was named pAd5/CMV-HSApac. This plasmid was then digested with HindIII and BamHI and the vector fragment was isolated and ligated
- 25 to the polylinker sequence obtained after digestion of AdMire with HindIII and BglII. The resulting plasmid was named pAdApt. Adapter plasmid pAdApt contains nucleotides -735 to +95 of the human CMV promoter (Boshart et al., 1985). A second version of this adapter plasmid containing a SalI
- 30 site in place of the PacI site upstream of the left ITR was made by inserting the 0.7 kb ScaI-BsrGI fragment from pAd5/Clipsal into pAdApt digested with ScaI and partially digested with BsrGI. This clone was named pAdApt.sal.

### *Generation of recombinant adenoviruses based on Ad5*

- RCA free recombinant adenoviruses can be generated very efficiently using the above described adapter plasmids and the pWe/Ad.AflIII-rITR or pWE/Ad.AflIII-rITrsp constructs.
- 5 Generally, the adapter plasmid containing the desired transgene in the desired expression cassette is digested with suitable enzymes to liberate the insert from vector sequences at the 3' and/or at the 5' end. The adenoviral complementation plasmids pWE/Ad.AflIII-rITR or pWE/Ad.AflIII-
- 10 rITRsp are digested with PacI to liberate the adeno sequences from the vector plasmids. As a non-limiting example the generation of AdApt-LacZ is described. Adapter plasmid pAdApt-LacZ was generated as follows. The E.coli LacZ gene was amplified from the plasmid pMLP.nlsLacZ (EP
- 15 95-202 213) by PCR with the primers 5'-GGGGTGGCCAGGGTACCTCTAGGCTTTTGCAA-3' and 5'-GGGGGGATCCATAAACAAGTTCAGAATCC-3'. The PCR reaction was performed with Ex Taq (Takara) according to the suppliers protocol at the following amplification program: 5 minutes
- 20 94°C, 1 cycle; 45 seconds 94°C and 30 seconds 60°C and 2 minutes 72°C, 5 cycles; 45 seconds 94°C and 30 seconds 65°C and 2 minutes 72°C, 25 cycles; 10 minutes 72; 45 seconds 94°C and 30 seconds 60°C and 2 minutes 72°C, 5 cycles, 1
- 25 cycle. The PCR product was subsequently digested with KpnI and BamHI and the digested DNA fragment was ligated into KpnI/BamHI digested pcDNA3 (Invitrogen), giving rise to pcDNA3.nlsLacZ. Construct pcDNA3.nlsLacZ was then digested with KpnI and BamHI and the 3 kb LacZ fragment was isolated from gel using the geneclean spin kit (Bio 101, Inc.).
- 30 pAdApt was also digested with KpnI and BamHI and the linear vector fragment was isolated from gel as above. Both isolated fragments were ligated and one clone containing the LacZ insert was selected. Construct pAdApt-LacZ was digested with SalI, purified by the geneclean spin kit and
- 35 subsequently digested with PacI. pWE/Ad.AflIII-rITRsp was digested with PacI. Both digestion mixtures were treated for

30' by 65 °C to inactivate the enzymes. Samples were put on gel to estimate the concentration.  $2.5 \times 10^6$  PER.C6 cells (ECACC deposit number 96022940) were seeded in T25 flasks in DMEM with 10% FCS and 10mM MgCl. The next day four microgram of each plasmid was transfected into PER.C6 cells (ECACC deposit number 96022940) using lipofectamine transfection reagent (Life Technologies Inc.) according to instructions of the manufacturer. The next day the medium was replaced by fresh culture medium and cells were further cultured at 37° C, 10% CO<sub>2</sub>. Again 24 hrs. later cells were trypsinised, seeded into T80 flasks and cultured at 37°C, 10% CO<sub>2</sub>. Full CPE was obtained 6 days after seeding in the T80 flask. Cells were harvested in the medium and subjected to one freeze/thaw cycle. The crude lysate obtained this way was used to plaque purify the mixture of viruses. Ten plaques were picked, expanded in a 24 well plate and tested for LacZ expression following infection of A549 cells. Viruses from all ten plaques expressed LacZ.

### 20 Example 3

#### Generation of chimeric recombinant adenoviruses

##### *Generation of hexon chimeric Ad5-based adenoviruses*

25 Neutralising antibodies in human serum are mainly directed to the hexon protein and to a lesser extent to the penton protein. Hexon proteins from different serotypes show highly variable regions present in loops that are predicted to be exposed at the outside of the virus (Athappilly et al., 1994; J. Mol. Biol. 242, 430-455). Most type specific epitopes have been mapped to these highly variable regions (Toogood et al., 1989; J. Gen Virol. 70, 3203-3214). Thus replacement of (part of ) the hexon sequences with corresponding sequences from a different serotype is an effective strategy to circumvent (pre-existing) neutralising

antibodies to Ad5. Hexon coding sequences of adenovirus serotype 5 are located between nucleotides 18841 and 21697. To facilitate easy exchange of hexon coding sequences from alternative adenovirus serotypes into the adenovirus

- 5 serotype 5 backbone, first a shuttle vector was generated. This subclone, coded pBr/Ad.Eco-PmeI, was generated by first digesting plasmid pBr322 with EcoRI and EcoRV and inserting the 14 kb PmeI-EcoRI fragment from pWE/Ad.AflIII-Eco. In this shuttle vector a deletion was made of a 1430 bp SanDI
- 10 fragment by digestion with SanDI and religation to give pBr/Ad.Eco-PmeI ΔSanDI. The removed fragment contains unique SpeI and MunI sites. From pBr/Ad.Eco-PmeIΔSanDI the adenovirus serotype 5 DNA encoding hexon was deleted. Hereto, the hexon flanking sequences were PCR amplified and
- 15 linked together thereby generating unique restriction sites replacing the hexon coding region. For these PCR reactions four different oligonucleotides were required: Δhex1-Δhex4.
- Δhex1: 5'- CCT GGT GCT GCC AAC AGC-3'
- Δhex2: 5'- CCG GAT CCA CTA GTG GAA AGC GGG CGC GCG-3'
- 20 Δhex3: 5'- CCG GAT CCA ATT GAG AAG CAA GCA ACA TCA ACA AC-3'
- Δhex4: 5'- GAG AAG GGC ATG GAG GCT G-3'

- The amplified DNA product of ± 1100 bp obtained with oligonucleotides Δhex1 and Δhex2 was digested with BamHI and FseI. The amplified DNA product of ± 1600 bp obtained with
- 25 oligonucleotides Δhex3 and Δhex4 was digested with BamHI and SbfI. These digested PCR fragments were subsequently purified from agarose gel and in a tri-part ligation reaction using T4 ligase enzyme linked to pBr/Ad.Eco-PmeI ΔSanDI digested with FseI and SbfI. The resulting construct
- 30 was coded pBr/Ad.Eco-PmeΔHexon. This construct was sequenced in part to confirm the correct nucleotide sequence and the presence of unique restriction sites MunI and SpeI. pBr/Ad.Eco-PmeΔHexon serves as a shuttle vector to introduce heterologous hexon sequences amplified from virus DNA from
- 35 different serotypes using primers that introduce the unique

restriction sites *MunI* and *SpeI* at the 5' and 3' ends of the hexon sequences respectively. To generate Ad5-based vectors that contain hexon sequences from the serotypes to which healthy individuals have no, or very low, titers of NAB the hexon sequences of Ad35, Ad34, Ad26 and Ad48 were amplified using the following primers:

Hex-up2: 5'-GACTAGTCAAGATGGCYACCCCHTCGATGATG-3' and

Hex-do2: 5'-GCTGGCCAATTGTTATGTRGTRGCGTTRCCGGC-3'.

These primers were designed using the sequences of published hexon coding regions (for example hexon sequences of Ad2, Ad3, Ad4, Ad5, Ad7, Ad16, Ad40 and Ad41 can be obtained at Genbank). Degenerated nucleotides were incorporated at positions that show variation between serotypes.

PCR products were digested with *SpeI* and *MunI* and cloned into the pBr/Ad.Eco-PmeΔHexon construct digested with the same enzymes.

The hexon modified sequences were subsequently introduced in the construct pWE/Ad.AflII-rITR by exchange of the *AscI* fragment generating pWE/Ad.AflII-rITRHexXX where XX stands for the serotype used to amplify hexon sequences.

The pWE/Ad.AflII-rITRHexXX constructs are then used to make viruses in the same manner as described above for Ad5 recombinant viruses.

25

#### *Generation of penton chimeric Ad5-based recombinant viruses*

The adenovirus type 5 penton gene is located between sequences 14156 and 15869. Penton base is the adenovirus capsid protein that mediates internalisation of the virus into the target cell. At least some serotypes (type C and B) have been shown to achieve this by interaction of an RGD sequence in penton with integrins on the cell surface. However, type F adenoviruses do not have an RGD sequence and for most viruses of the A and D group the penton sequence is not known. Therefore, penton may be involved in target cell specificity. Furthermore, as a capsid protein, the penton

protein is involved in the immunogenicity of the adenovirus (Gahery-Segard et al., 1998). Therefore, replacement of Ad5 penton sequences with penton sequences from serotypes to which no or low titers of NAB exist in addition to

- 5 replacement of the hexon sequences will prevent clearance of the adenoviral vector more efficiently than replacement of hexon alone. Replacement of penton sequences may also affect infection specificity.

To be able to introduce heterologous penton sequences in Ad5  
10 we made use of the plasmid-based system described above.

- First a shuttle vector for penton sequences was made by insertion of the 7.2 kb *NheI*-*EcoRV* fragment from construct pWE/Ad.AflIII-*EcoRI* into pBr322 digested with the same enzymes. The resulting vector was named pBr/XN. From this  
15 plasmid Ad5 penton sequences were deleted and replaced by unique restriction sites that are then used to introduce new penton sequences from other serotypes. Hereto, the left flanking sequences of penton in pBr/XN were PCR amplified using the following primers:

- 20 DP5-F: 5'- CTG TTG CTG CTG CTA ATA GC-3' and  
DP5-R: 5'- CGC GGA TCC TGT ACA ACT AAG GGG AAT ACA AG-3'  
DP5-R has an *Bam*HI site (underlined) for ligation to the right flanking sequence and also introduces a unique *Bsr*GI site (bold face) at the 5'-end of the former Ad5 penton  
25 region.

The right flanking sequence was amplified using:

- DP3-F: 5'-CGC GGA TCC CTT AAG GCA AGC ATG TCC ATC CTT-3' and  
DP3-3R: 5'- AAA ACA CGT TTT ACG CGT CGA CCT TTC-3'  
DP3-F has an *Bam*HI site (underlined) for ligation to the  
30 left flanking sequence and also introduces a unique *Afl*III site (bold face) at the 3'-end of the former Ad5 penton region.

- The two resulting PCR fragments were digested with *Bam*HI and ligated together. Then this ligation mixture was digested  
35 with *Avr*II and *Bgl*III. pBr/XN was also digested with *Avr*II



and BglII and the vector fragment was ligated to the digested ligated PCR fragments. The resulting clone was named pBr/Ad.Δpenton. Penton coding sequences from Ad35, Ad34, Ad26 and Ad48 were PCR amplified such that the 5' and 3' ends contained the BsrGI and AflIII sites respectively. Hereto, the following primers were used:

For Ad34 and Ad35:

P3-for: 5'-GCT CGA TGT ACA ATG AGG AGA CGA GCC GTG CTA-3'

P3-rev: 5'-GCT CGA CTT AAG TTA GAA AGT GCG GCT TGA AAG-3'

10 For Ad26 and Ad48:

P17F: 5'-GCT CGA TGT ACA ATG AGG CGT GCG GTG GTG TCT TC-3'

P17R: 5'-GCT CGA CTT AAG TTA GAA GGT GCG ACT GGA AAG C-3'

Amplified pcr products were digested with BfrI and BsrGI and cloned into pBr/Ad.Δpenton digested with the same enzymes. Introduction of these heterologous penton sequences in pBr/Ad.Δpenton generated constructs named pBr/Ad.pentonXX where XX represents the number of the serotype corresponding to the serotype used to amplify the inserted penton sequences. Subsequently the new penton sequences were introduced in the a pWE/Ad.AflIII-rITR vector having a modified hexon. For example penton sequences from Ad35 were introduced in the construct pWE/Ad.AflII-rITRHex35 by exchange of the common PseI fragment. Other combinations of penton and hexon sequences were also made. Viruses with modified hexon and penton sequences were made as described above using cotransfection with an adapter plasmid on PER.C6 cells (ECACC deposit number 96022940). In addition, penton sequences were introduced in the pWE/Ad.AflII-rITR construct. The latter constructs contain only a modified penton and viruses generated from these constructs will be used to study the contribution of penton sequences to the neutralisation of adenoviruses and also for analysis of possible changes in infection efficiency and specificity.

### Generation of fiber chimeric Ad5-based viruses

Adenovirus infection is mediated by two capsid proteins fiber and penton. Binding of the virus to the cells is achieved by interaction of the protruding fiber protein with a receptor on the cell surface. Internalisation then takes place after interaction of the penton protein with integrins on the cell surface. At least some adenovirus from subgroup C and B have been shown to use a different receptor for cell binding and therefore have different infection efficiencies on different cell types. Thus it is possible to change the infection spectrum of adenoviruses by changing the fiber in the capsid. The fiber coding sequence of adenovirus serotype 5 is located between nucleotides 31042 and 32787. To remove the adenovirus serotype 5 DNA encoding fiber we started with construct pBr/Ad.Bam-rITR. First a NdeI site was removed from this construct. For this purpose, pBr322 plasmid DNA was digested with NdeI after which protruding ends were filled using Klenow enzym. This pBr322 plasmid was then religated, digested with NdeI and transformed into *E.coli* DH5 $\alpha$ .

The obtained pBr/ $\Delta$ NdeI plasmid was digested with ScaI and SalI and the resulting 3198 bp vector fragment was ligated to the 15349 bp ScaI-SalI fragment derived from pBr/Ad.BamrITR, resulting in plasmid pBr/Ad.Bam-rITR $\Delta$ NdeI which hence contained a unique NdeI site. Next a PCR was performed with oligonucleotides NY-up:

5'- CGA CAT ATG TAG ATG CAT TAG TTT GTG TTA TGT TTC AAC GTG-3'

and

NY-down:

5'-GGA GAC CAC TGC CAT GTT-3'

During amplification, both a NdeI (bold face) and a NsiI restriction site (underlined) were introduced to facilitate cloning of the amplified fiber DNAs. Amplification consisted of 25 cycles of each 45 sec. at 94°C, 1 min. at 60°C, and 45 sec. at 72°C. The PCR reaction contained 25 pmol of oligonucleotides NY-up or NY-down, 2mM dNTP, PCR buffer with

1.5 mM MgCl<sub>2</sub>, and 1 unit of Elongase heat stable polymerase (Gibco, The Netherlands). One-tenth of the PCR product was run on an agarose gel which demonstrated that the expected DNA fragment of  $\pm$  2200 bp was amplified. This PCR fragment was subsequently purified using Geneclean kit system (Bio101 Inc.). Then, both the construct pBr/Ad.Bam-rITRANdeI as well as the PCR product were digested with restriction enzymes NdeI and SbfI. The PCR fragment was subsequently cloned using T4 ligase enzyme into the NdeI and SbfI digested pBr/Ad.Bam-rITRANdeI, generating pBr/Ad.BamRAFIb. This plasmid allows insertion of any PCR amplified fiber sequence through the unique NdeI and NsiI sites that are inserted in place of the removed fiber sequence. Viruses can be generated by a double homologous recombination in packaging cells described in patent No. PCT/NL96/00244 using an adapter plasmid, construct pBr/Ad.AflIII-EcoRI digested with PacI and EcoRI and a pBr/Ad.BamRAFIb construct in which heterologous fiber sequences have been inserted. To increase the efficiency of virus generation, the construct pBr/Ad.BamRAFIb was modified to generate a PacI site flanking the right ITR. Hereto, pBr/Ad.BamRAFIb was digested with AvrII and the 5 kb adenofragment was isolated and introduced into the vector pBr/Ad.Bam-rITR.pac#8 described above replacing the corresponding AvrII fragment. The resulting construct was named pBr/Ad.BamRAFIb.pac. Once a heterologous fiber sequence is introduced in pBr/Ad.BamRAFIb.pac, the fiber modified right hand adenovirus clone is introduced into a large cosmid clone as described above for pWE/Ad.AflIII-rITR. Such a large cosmid clone allows generation of adenovirus by only one homologous recombination. Ad5-based viruses with modified fibers have been made and described (nos. 98204482.8 and 99200624.7). In addition, hexon and penton sequences from serotypes from this invention are combined with the desired fiber sequences to generate viruses which infect the target cell of choice

very efficiently. For example smooth muscle cells, endothelial cells or synoviocytes all from human origin are very well infected with Ad5 based viruses with a fiber from subgroup B viruses especially adenovirus type 16.

- 5 The above described examples in which specific sequences can be deleted from the Ad5 backbone in the plasmids and replaced by corresponding sequences from other serotypes clearly show the flexibility of the system. It is evident that by the methods described above any combination of
- 10 capsid gene from different serotypes can be made. Thus, chimeric recombinant Ad5-based adenoviruses are designed with desired hexon and penton sequences making the virus less sensitive for neutralisation and with desired fiber sequences allowing efficient infection in specific target
- 15 tissues.

#### Example 4

##### Construction of a plasmid-based system to generate Ad35 recombinant viruses

20

Partial restriction maps of Ad35 have been published previously (Valderrama-Leon *et al.*, 1985; Kang *et al.*, 1989; Li *et al.* 1991). An example of a functional plasmid-based

25 system to generate recombinant adenoviruses based on Ad35 consists of the following elements:

1. An adapter plasmid comprising a left ITR and packaging sequences derived from Ad35 and at least one restriction site for insertion of an heterologous expression cassette and lacking E1 sequences. Furthermore, the adapter
- 30 plasmid contains Ad35 sequences 3' from the E1B coding region including the pIX promoter and coding sequences sufficient to mediate homologous recombination of the adapter plasmid with a second nucleotide.
- 35 2. A second nucleotide comprising sequences homologous to the adapter plasmid and Ad35 sequences necessary for the

replication and packaging of the recombinant virus, that is early, intermediate and late genes that are not present in the packaging cell.

3. A packaging cell providing at least functional E1 proteins capable of complementing the E1 function of Ad35.

Ad35 DNA was isolated from a purified virus batch as follows. To 100  $\mu$ l of virus stock (Ad35:  $3.26 \times 10^{12}$  VP/ml) 10  $\mu$ l 10x DNase buffer (130 mM Tris-HCl pH7.5; 1,2 M CaCl<sub>2</sub>; 50mM MgCl<sub>2</sub>) was added. After addition of 10  $\mu$ l 10mgr/ml DNase I (Roche Diagnostics) the mixture was incubated for 1 hr. at 37°C. Following addition of 2.5  $\mu$ l 0.5M EDTA, 3.2  $\mu$ l 20% SDS and 1.5  $\mu$ l ProteinaseK (Roche Diagnostics; 20mgr/ml) samples were incubated at 50°C for 1 hr. Next, the viral DNA was isolated using the Geneclean spin kit (Bio101 Inc.) according to the manufacturers instructions. DNA was eluted from the spin column with 25  $\mu$ l sterile MilliQ water. In the following sizes of DNA fragments and fragment numbering will be used according to Kang et al. (1989). Ad35 DNA was digested with EcoRI and the three fragments (approximately 22.3 (A), 7.3 (B) and 6 kb (C)) were isolated from gel using the Geneclean kit (Bio101, Inc.). pBr322 was digested with EcoRI or with EcoRI and EcoRV and digested fragments were isolated from gel and dephosphorylated with Tsap enzyme (Gibco BRL). Next, the 6 kb Ad35 C fragment was ligated to the pBr322xEcoRI fragment and the ITR-containing Ad35 fragment (EcoRI-B) was ligated to the pBr322xEcoRI/EcoRV fragment. Ligations were incubated at 16°C overnight and transformed into DH5 $\alpha$  competent bacteria (Life Techn.). Minipreps of obtained colonies were analysed for correct insertion of the Ad35 fragments by restriction analysis. Both the 6 kb and the 7.3 kb Ad35 fragment were found to be correctly inserted in pBr322. The 6kb fragment was isolated in both orientations pBr/Ad35-Eco6.0<sup>+</sup> and pBr/Ad35-Eco6.0<sup>-</sup> whereby the + stands for 5' to 3'

orientation relative to pBr322. The clone with the 7.3 kb Ad35 B insert, named pBr/Ad35-Eco7.3 was partially sequenced to check correct ligation of the 3' ITR. It was found that the ITR had the sequence 5'- CATCATCAAT~~3~~-3' in the lower strand. Then pBr/Ad35-Eco7.3 was extended to the 5' end by insertion of the 6kb Ad35 fragment. Hereto, pBr/Ad35-Eco7.3 was digested with EcoRI and dephosphorylated. The fragment was isolated from gel and ligated to the 6kb Ad35 EcoRI fragment. After transformation clones were tested for correct orientation of the insert and one clone was selected, named pBr/Ad35-Eco13.3.

This clone is then extended with the ~5.4 kb SalI D fragment obtained after digestion of wt Ad35 with SalI. Hereto, the SalI site in the pBr322 backbone is removed by partial digestion of pBr/Ad35-Eco13.3 with SalI, filling in of the sticky ends by Klenow treatment and religation. One clone is selected that contains a single SalI site in the adenoviral insert. This clone, named pBr $\Delta$ sal /Ad35-Eco13.3 is then linearised with AatII which is present in the pBr322 backbone and ligated to a SalI linker with AatII complementary ends. The DNA is then digested with excess SalI and the linear fragment is isolated and ligated to the 5.4 kb SalI-D fragment from Ad35. One clone is selected that contains the SalI fragment inserted in the correct orientation in pBr/Ad35-Eco13.3. The resulting clone, pBr/Ad35.Sal2-rITR contains the 3' ~17 kb of Ad35 including the right ITR. To enable liberation of the right ITR from the vector sequences at the time of virus generation, a NotI site flanking the right ITR is introduced by PCR.

The Ad35 EcoRI-A fragment of 22.3 kb was also cloned in pBr322xEcoRI/EcoRV. One clone, named pBr/Ad35-EcoA3', was selected that apparently had a deletion of approximately 7kb of the 5' end. It did contain the SalI site at 9.4 kb in Ad35 wt DNA and approximately 1.5 kb of sequences upstream.

Using this SalI site and the unique NdeI site in the pBr322 backbone this clone is extended to the 5' end by insertion

of an approximately 5 kb Ad35 fragment 5' from the first SalI in Ad35 in such a way that a NotI restriction site is created at the 5' end of the Ad35 by insertion of a linker. This clone, named pBr/Ad35.pIX-EcoA does not contain the  
5 left end sequences (ITR, packaging sequences and E1) and at the 3' end it has approximately 3.5 kb overlap with clone pBr/Ad35.Sal2-rITR.

To create an adapter plasmid, Ad35 was digested with SalI and the left end B fragment of ~9.4 kb was isolated. pBr322  
10 was digested with EcoRV and SalI, isolated from gel and dephosphorylated with Tsap enzyme. Both fragments are ligated and clones with correct insertion and correct sequence of the left ITR are selected. To enable liberation of the left ITR from the vector sequences at the time of  
15 virus generation, a NotI site flanking the left ITR is introduced by PCR. From this clone the E1 sequences are deleted and replaced by a polylinker sequence using PCR. The polylinker sequence is used to introduce an expression cassette for a gene of choice.

20 Recombinant Ad35 clones are generated by transfection of PER.C6 cells with the adapter plasmid, pBr/Ad35.pIX-EcoA and pBr/Ad35.Sal2-rITR as shown in figure 3. Homologous recombination gives rise to recombinant viruses.

Legend to the figures:

## Figure 1:

- 5 Bar graph showing the percentage of serum samples positive for neutralisation for each human wt adenovirus tested (see example1 for description of the neutralisation assay).

10

## Figure 2:

Graph showing absence of correlation between the VP/CCID50 ratio and the percentage of neutralisation.

- 15 Figure 3:

Schematic representation of a partial restriction map of Ad35 (taken from Kang et al., 1989) and the clones generated to make recombinant Ad35-based viruses.

20

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Table I:

Serotype	Elution [NaCl] mM	VP/ml	CCID50	log <sub>10</sub> VP/CCID50 ratio
1	597	8.66x10 <sup>10</sup>	5.00x10 <sup>7</sup>	3.2
2	574	1.04x10 <sup>12</sup>	3.66x10 <sup>11</sup>	0.4
3	131	1.19x10 <sup>11</sup>	1.28x10 <sup>7</sup>	4.0
4	260	4.84x10 <sup>11</sup>	2.50x10 <sup>8</sup>	3.3
5	533	5.40x10 <sup>11</sup>	1.12x10 <sup>10</sup>	1.7
6	477	1.05x10 <sup>12</sup>	2.14x10 <sup>10</sup>	1.7
7	328	1.68x10 <sup>12</sup>	2.73x10 <sup>9</sup>	2.4
9	379	4.99x10 <sup>11</sup>	3.75x10 <sup>7</sup>	4.1
10	387	8.32x10 <sup>12</sup>	1.12x10 <sup>9</sup>	3.9
12	305	3.64x10 <sup>11</sup>	1.46x10 <sup>7</sup>	4.4
13	231	4.37x10 <sup>12</sup>	7.31x10 <sup>8</sup>	3.8
15	443	5.33x10 <sup>12</sup>	1.25x10 <sup>9</sup>	3.6
16	312	1.75x10 <sup>12</sup>	5.59x10 <sup>8</sup>	3.5
17	478	1.39x10 <sup>12</sup>	1.45x10 <sup>9</sup>	3.0
19	430	8.44x10 <sup>11</sup>	8.55x10 <sup>7</sup>	4.0
20	156	1.41x10 <sup>11</sup>	1.68x10 <sup>7</sup>	3.9
21	437	3.21x10 <sup>11</sup>	1.12x10 <sup>8</sup>	3.5
22	365	1.43x10 <sup>12</sup>	5.59x10 <sup>7</sup>	3.4
23	132	2.33x10 <sup>11</sup>	1.57x10 <sup>7</sup>	4.2
24	405	5.12x10 <sup>12</sup>	4.27x10 <sup>8</sup>	4.1
25	405	7.24x10 <sup>11</sup>	5.59x10 <sup>7</sup>	4.1
26	356	1.13x10 <sup>12</sup>	1.12x10 <sup>9</sup>	4.0
27	342	2.00x10 <sup>12</sup>	1.28x10 <sup>8</sup>	4.2
28	347	2.77x10 <sup>12</sup>	5.00x10 <sup>7</sup>	4.7
29	386	2.78x10 <sup>11</sup>	2.00x10 <sup>7</sup>	4.1
30	409	1.33x10 <sup>12</sup>	5.59x10 <sup>8</sup>	3.4
31	303	8.48x10 <sup>10</sup>	2.19x10 <sup>7</sup>	3.6
33	302	1.02x10 <sup>12</sup>	1.12x10 <sup>7</sup>	5.0
34	425	1.08x10 <sup>12</sup>	1.63x10 <sup>11</sup>	0.8
35	446	3.26x10 <sup>12</sup>	1.25x10 <sup>11</sup>	1.4
36	325	9.26x10 <sup>12</sup>	3.62x10 <sup>9</sup>	3.4
37	257	5.86x10 <sup>12</sup>	2.8x10 <sup>9</sup>	3.3
38	337	3.61x10 <sup>12</sup>	5.59x10 <sup>7</sup>	4.8
39	241	3.34x10 <sup>11</sup>	1.17x10 <sup>7</sup>	4.5
42	370	1.95x10 <sup>12</sup>	1.12x10 <sup>8</sup>	4.2

Continued on next page.

Serotype #	Elution [NaCl] mM	VP/ml	CCID50	log <sub>10</sub> VP/CCID50 ratio
43	284	2.42x10 <sup>12</sup>	1.81x10 <sup>8</sup>	4.1
44	295	8.45x10 <sup>11</sup>	2.00x10 <sup>7</sup>	4.6
45	283	5.20x10 <sup>11</sup>	2.99x10 <sup>7</sup>	4.2
46	282	9.73x10 <sup>12</sup>	2.50x10 <sup>8</sup>	4.6
47	271	5.69x10 <sup>11</sup>	3.42x10 <sup>7</sup>	4.2
48	264	1.68x10 <sup>12</sup>	9.56x10 <sup>6</sup>	3.3
49	332	2.20x10 <sup>12</sup>	8.55x10 <sup>7</sup>	4.4
50	459	7.38x10 <sup>12</sup>	2.80x10 <sup>8</sup>	3.4
51	450	8.41x10 <sup>11</sup>	1.88x10 <sup>8</sup>	3.7

Legend to table I:

- 5 All human adenoviruses used in the neutralisation experiments were produced on PER.C6 cells (ECACC deposit number 96022940) (Fallaux et al., 1998) and purified on CsCl as described in example 1. The NaCl concentration at which the different serotypes eluted from the HPLC column is shown. Virus particles/ml (VP/ml) were calculated from an Ad5 standard.
- 10 The titer in the experiment (CCID50) was determined on PER.C6 cells (ECACC deposit number 96022940) as described in example 1 by titrations performed in parallel with the neutralisation experiment. The CCID50 is shown for the 44 viruses used in this study and reflects the dilution of the virus needed to
- 15 obtain CPE in 50% of the wells after 5 days. The ratio of VP/CCID50 is depicted in log<sub>10</sub> and is a measurement of the infectivity of the different batches on PER.C6 cells (ECACC deposit number 96022940).

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CLAIMS

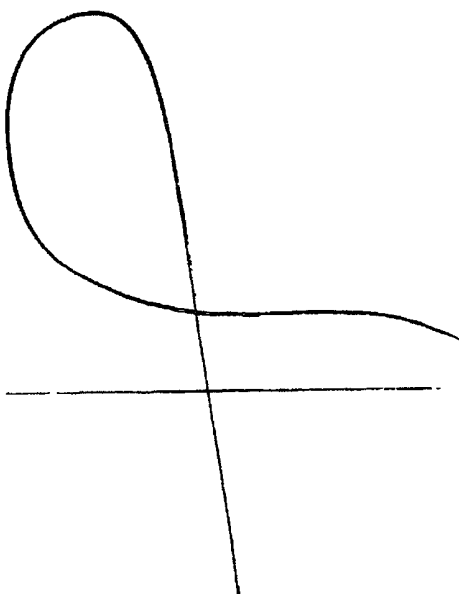
(58)

1. A gene delivery vehicle comprising at least one of the adenovirus serotype 35 elements or a functional equivalent thereof, responsible for avoiding or diminishing neutralising activity against adenoviral elements by the host to which the gene is to be delivered and a gene of interest.
2. Adenovirus serotype 35 or a functional homologue thereof or a chimaeric virus derived therefrom, or a gene delivery vehicle based on said virus its homologue or its chimaera for use as a pharmaceutical.
3. A gene delivery vehicle according to claim 1, whereby said elements comprise adenovirus 35 E3 expression products or the genes encoding them.
4. A gene delivery vehicle according to claim 1 or 3, whereby said elements comprise adenovirus 35 fiber, penton and/or hexon proteins or a gene encoding either.
5. A gene delivery according to any one of claims 1, 3, or 4 which is a chimaera of adenovirus 35 with at least one other adenovirus.
- ~~6. A gene delivery vehicle according to any one of claims~~
- 1, 3, 4 or 5 which has a different tropism than adenovirus 35.
7. A nucleic acid encoding at least a functional part of a gene delivery vehicle according to any one of claims 1 or 3-6, or a virus, homologue or chimaera thereof according to claim 2.
8. A nucleic acid encoding at least one of the adenovirus serotype 35 elements or a functional equivalent thereof, responsible for avoiding or diminishing neutralising activity against adenoviral elements by the host to which the gene is to be delivered and having a site for introducing a gene of interest therein.
9. A nucleic acid according to claim 7 or 8, further comprising at least one ITR.

10. A nucleic acid according to claim 7, 8 or 9 further comprising a region of nucleotides designed or useable for homologous recombination.
11. At least one set of two nucleic acids comprising a  
5 nucleic acid according to any one of claims 7-10, whereby said set of nucleic acids is capable of a single homologous recombination event with each other, which leads to a nucleic acid encoding a functional gene delivery vehicle.
12. A cell comprising a nucleic acid according to any one of  
10 claims 7-10 or a set of nucleic acids according to claim 11.
13. A cell according to claim 12 which complements the necessary elements for adenoviral replication which are absent from the nucleic acid according to any one of claims 7-10 or a set of nucleic acids according to claim 11.
14. A cell according to claim 12 or 13, which originates  
15 from a PER.C6 cell (ECACC deposit number 96022940).
15. A method for producing a gene delivery vehicle according to claim 1, or 3-6, comprising expressing a nucleic acid according to any one of claims 7-10 in a cell according to  
20 claim 12 or 13 and harvesting the resulting gene delivery vehicle.
- 
16. A method for producing a gene delivery vehicle according to claim 1, or 3-6, comprising culturing a cell according to claim 12 or 13 in a suitable culture medium and harvesting  
25 the resulting gene delivery vehicle.
17. A gene delivery vehicle obtainable by a method according to claims 15 or 16.
18. A gene delivery vehicle according to any one of claims 1,3-6 or 17, which is derived from a chimaera of an  
30 adenovirus and an integrating virus.
19. A gene delivery vehicle according to claim 18, wherein said integrating virus is adeno associated virus.
20. A gene delivery vehicle according to any one of claims 1, 3-6 or 17-19, which has the tropism determining parts of  
35 adenovirus 16 or functional equivalents thereof.



21. A gene delivery vehicle according to anyone of claims 1, 3-6 or 17-20 for use as a pharmaceutical.
22. A pharmaceutical formulation comprising a gene delivery vehicle according to any one of claims 1, 3-6 or 17-20 and a  
5 suitable excipient.
23. A pharmaceutical formulation comprising an adenovirus, a chimaera thereof, or a functional homologue thereof according to claim 2 and a suitable excipient.

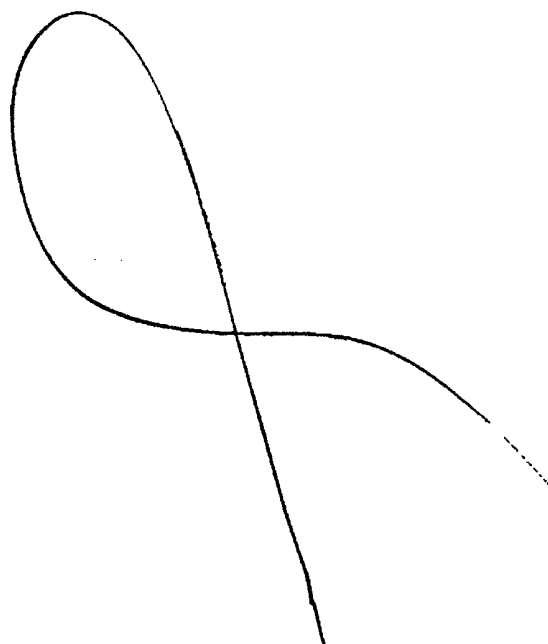


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ABSTRACT

(58)

The serotypes differ in their natural tropism. The adenovirus serotypes 2, 4, 5 and 7 all have a natural affiliation towards lung epithelia and other respiratory tissues. In contrast, serotypes 40 and 41 have a natural affiliation towards the gastrointestinal tract. The serotypes described above, differ in at least capsid proteins (penton-base, hexon), proteins responsible for cell binding (fiber protein), and proteins involved in adenovirus replication. This difference in tropism and capsid protein among serotypes has led to the many research efforts aimed at redirecting the adenovirus tropism by modification of the capsid proteins (REF).



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Figure 1:  
% of human sera with neutralising capacity for human adenovirus (n=100)

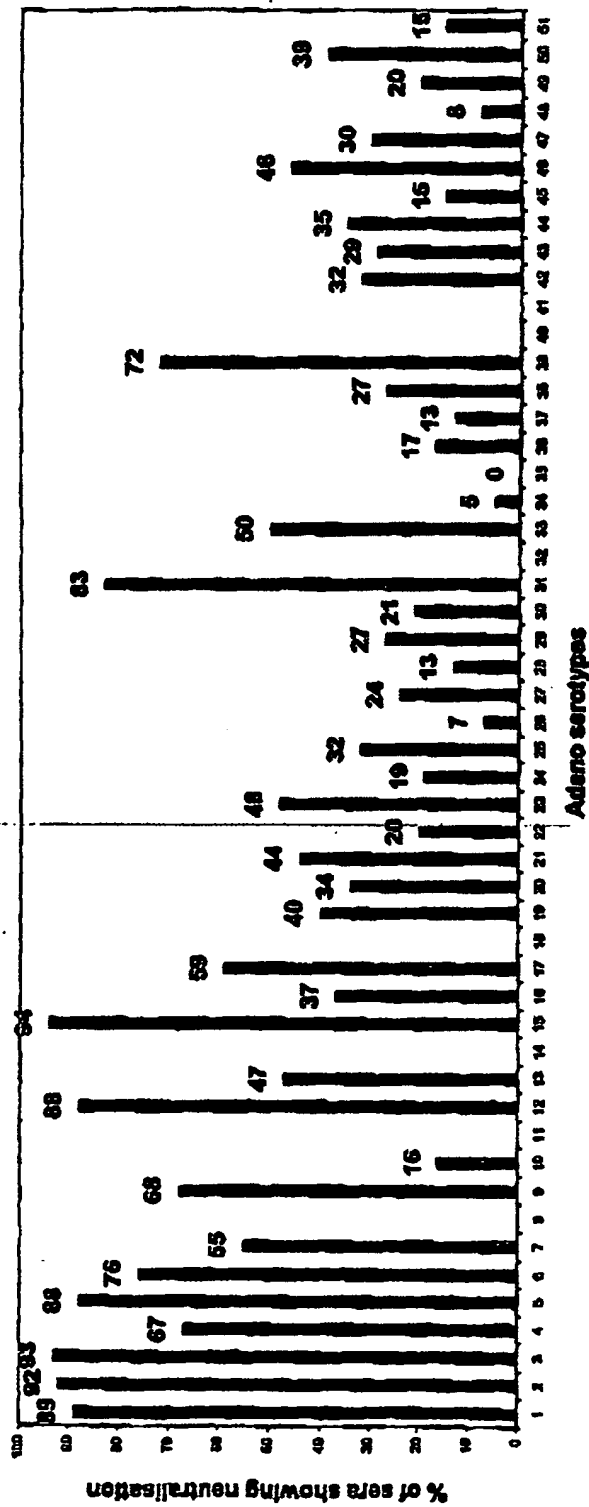


Figure 2

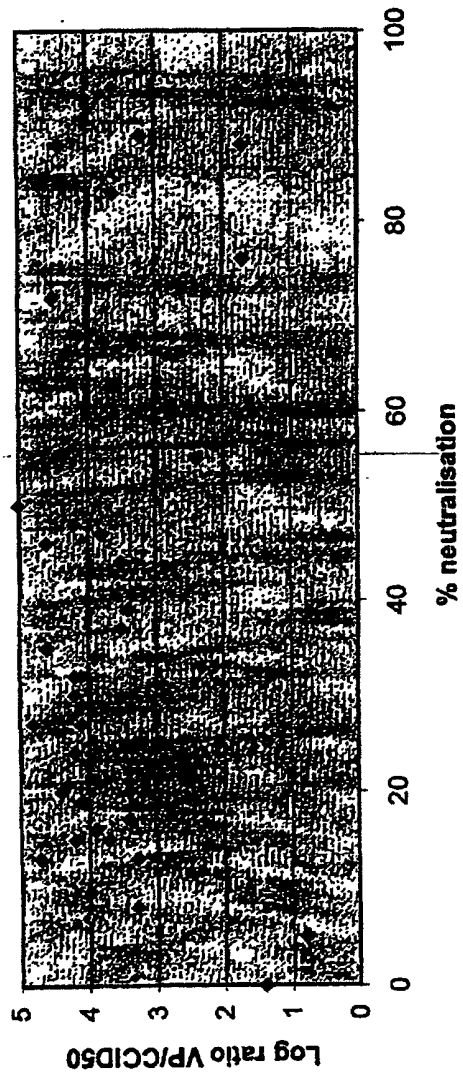
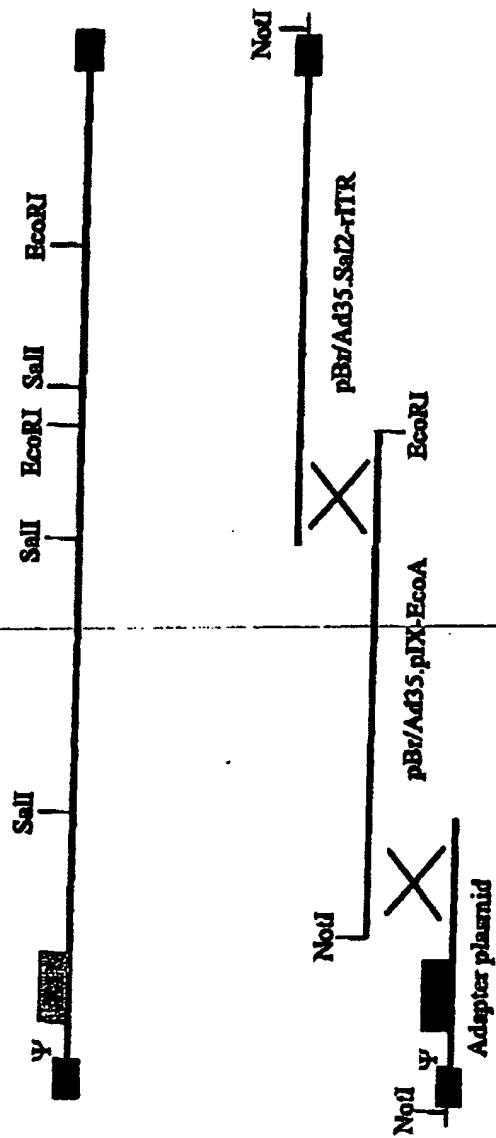
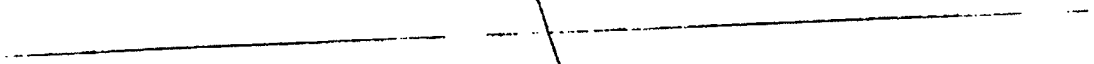
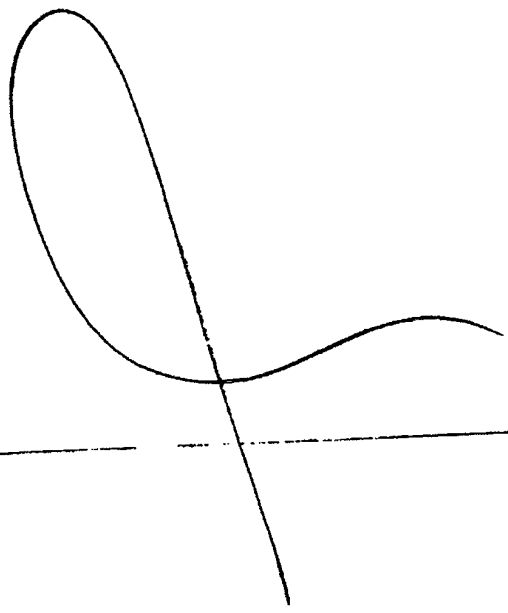


Figure 3: Ad35 plasmid-based system for virus generation







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